R93-16

Comp. Biochem. Physiol. Vol. 105B, Nos 3/4, pp. 487–496, 1993
Printed in Great Britain

0305-0491/93 $6.00 + 0.00
Pergamon Press Ltd

COMPARATIVE ANALYSIS OF MACROMOLECULES IN MOLLUSC SHELLS

J. KEITH,*† S. STOCKWELL,* D. BALL,* K. REMILLARD,* D. KAPLAN,*‡ T. THANNHAUSER§ and R. SHERWOOD§

*Biotechnology Division, U.S. Army Natick Research, Development and Engineering Center, Natick, MA 01760-5020 U.S.A. (Tel. 508 651-5525; Fax 508 651-5521); and §Analytical and Synthesis Facility, Cornell University Biotechnology Core Facility, Ithaca, NY 14853, U.S.A.

(*Received* 18 *December* 1992; *accepted* 5 *February* 1993)

Abstract—1. Proteins and polysaccharides were isolated from the shells of molluscs; blue mussel, *Mytilus edulis*, chambered nautilus, *Nautilus pompilius*, and red abalone, *Haliotus rufescens*.

2. *N*-acetyl glucosamine was detected in nautilus but not mussel or abalone.

3. Amino acid analysis of protein fractions was completed for the three molluscs and purified proteins from the mussel were partially sequenced.

4. Calcium binding studies were carried out with some of the protein fractions.

INTRODUCTION

The formation of molluscan shell structures or composites containing calcium carbonate involves control over crystal morphology, size, density, and orientation at the molecular level that is at present unattainable synthetically. The organic portion of most mollusc shells, consisting of 0.1–5% by weight of the total structure (Hare and Abelson, 1965), is postulated to be the source of this control (Wilbur, 1964; Krampitz *et al.*, 1976; Lowenstam and Weiner, 1989; Wheeler and Sikes, 1989).

A review of the literature shows a variety of compositions and functionalities assigned to this organic portion, termed the matrix, including proteins; specifically proteoglycans (Weiner *et al.*, 1983), phenyloxidase crosslinked proteins (Gordon and Carriker, 1980), and phosphoproteins (Sikes and Wheeler, 1983; Swift *et al.*, 1986; Veis *et al.*, 1986), polysaccharides; specifically chitin (Jeuniaux, 1963; Peters, 1972), sulfated mucopolysaccharides (Simkiss, 1965; Crenshaw and Ristedt, 1976), and neutral and amino sugars (Hare and Abelson, 1965; Crenshaw, 1972); and lipids (Beedham, 1958). Characterization of the matrix composition varies with species of mollusc (Hare and Abelson, 1965; Meenakshi *et al.*, 1971; Weiner and Hood, 1975; Krampitz *et al.*, 1976) and methodology (Krampitz *et al.*, 1983b). Attempts to characterize this organic matrix have focused on a soluble proteinaceous fraction with acidic functionality isolated mainly from *Mercenaria mercenaria*, *Crassostrea virginica*, or *Mytilus californianus* (Crenshaw, 1972; Krampitz *et al.*, 1976; Weiner, 1981) which inhibits crystallization (Weiner and Hood, 1975; Weiner, 1981; Sikes and Wheeler, 1983).

To begin to understand the mechanisms involved in the biomineralization process, a detailed analysis of the organic matrix of three molluscs, *Mytilus edulis*, *Nautilus pompilius* and *Haliotus rufescens* was performed. *M. edulis* was, in part, chosen for the complexity of the shell, which consists of an inner nacreous layer of aragonite, a pallial calcitic layer, and an outer prismatic layer of calcite (Gregoire, 1961, 1972; Kennedy *et al.*, 1969; Carriker, 1978). Comparisons were made with partially analyzed matrices of *N. pompilius* and *H. rufescens* isolated in the same way. The nautilus shell structure lacks the pallial calcitic layer and the outer prismatic layer, containing, instead, an outer aragonitic layer.

The objective of this work was to analyze the organic macromolecules, including polysaccharides and individual proteins from the organic matrix isolated from these molluscs. This analysis was to help us begin to understand relationships between protein and polysaccharide structure and mineral binding function through comparison with other species having different shell structures. Amino acid composition, *N*-terminal sequence, glycoprotein analysis, and polysaccharide composition were assessed using microanalytical methods.

MATERIALS AND METHODS

Matrix purification

Live specimens of *M. edulis* were collected locally (Marshfield, MA), maintained in seawater and processed within 24 hr of collection. Soft tissue was removed, the shells were scrubbed with 5% NaOH to remove the periostracum and any adhering organisms and then lightly crushed in a mortar and pestle. The

†Present address: Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, U.S.A.
‡To whom correspondence should be addressed.

crushed shell was ground to a fine powder in a freezer mill under liquid nitrogen and stored at −70°C. *N. pompilus* shell was provided by M. Sarikya (University of Washington, Seattle) and *H. rufescens* was purchased from the Abalone Farm (Cuyurcus, CA). The abalone was treated in a similar fashion to the mussel. The nautilus extracts were prepared from previously dried shell.

Extractions were carried out over 3 days by stirring 40 g of powdered shell at room temperature in two liters of 5% acetic acid under vacuum, according to Furlong and Humbert (1992) and the supernatant discarded after determining there was no protein present using a bicinchoninic acid assay (Smith *et al.*, 1985). The pellet was redissolved in 10 ml of Milli-Q® water and spun 3–5 min at 5000 rpm to separate the soluble matrix (supernatant) from the insoluble matrix (pellet). The supernatant and pellet were then transferred to separate microfuge tubes and dried in a Speed-Vac (Savant Instruments, Farmingdale, NY). This process rendered some of the previously soluble material insoluble.

Matrix characterization

Protein content of the purified soluble matrix material was determined by bicinchoninic acid assay. Protein content of purified insoluble matrix material was determined by elemental carbon, hydrogen, and nitrogen analysis performed by Oneida Research Services (Whitesboro, NY).

Polyacrylamide gel electrophoresis (PAGE) and Western blotting to PVDF membranes were performed on a Novex® Gel Electrophoresis system (Novel Experimental Technology, Encinitas, CA), with Novex® Tris–Gly and Novex® Tricine buffers and gradient gels used for PAGE and Novex® Carbonate, Novex® Tris–Gly buffer, and a calcium rich transfer buffer (McKeon and Lyman, 1991) used for Western transfer. The 95 kDa protein only transferred well in a semi-dry blotting apparatus manufactured by Integrated Separation Systems (Natick, MA) using their gradient buffer system. In an attempt to identify calcium binding proteins, SDS–PAGE gels were also stained with Stains-All (1-ethyl-2-(3-[1-ethylnaphtho(1,2-*d*)-thiazolin-2-ylidene]-2-methylpropenyl)naphtho(1,2-*d*)-thiazoliumbromide) (Sigma Chemical Co., St Louis, MO) (Campbell *et al.*, 1983), or with Alcian Blue (Sigma) to identify phosphoproteins (Butler *et al.*, 1981).

Amino acid analysis of the purified matrix, as well as protein bands purified by PAGE and Western transfer, was performed by derivatization after hydrolysis, and reverse-phase HPLC using the Waters Pico-Tag® method (Waters, Division of Millipore, Milford, MA). Analysis of individual protein bands transfered to PVDF membranes was by the method of LeGendre and Matsudaira (1988), while the standard manufacturer's protocols (Cohen *et al.*, 1989) were used for the total, soluble, and insoluble matrix proteins of both the mussel and nautilus. Controls consisting of a section of membrane without protein were run with each sample to correct for background.

Protein bands purified by PAGE and Western transfer were also *N*-terminally sequenced by Edman degradation on an ABI 470A gas phase sequencer (Applied Biosystems Inc., Foster City, CA) using the Blott cartridge. A polyclonal antibody (provided by Steve Benson, University of California, Hayward, CA) to the sea urchin spicule matrix was used to screen the isolated protein fractions.

Glycosylation of purified matrix proteins was determined by microanalysis by gas–liquid chromatography (GLC), based on the method of Chaplin (1982). A 20 μl aliquot of 0.001 M mesoinositol was added to <1 mg of material, either shell powder, purified matrices or protein bands excised from PVDF membranes, and the mixtures dried over P_2O_5 in a vacuum chamber with aspiration. A 3% methanolic hydrogen chloride solution, 150 μl, was added and the mixtures were stirred magnetically and heated at 70°C overnight in a Reacti-Therm Heating/Stirring Module in vials capped with Teflon-lined septa. T-butanol, 30 μl, was added to each vial and volatiles were removed with a stream of oxygen-free nitrogen at room temperature. To ensure complete *N*-acetylation of any amino sugars present, re-*N*-acetylation was carried out by addition of methanol, 150 μl; pyridine, 15 μl; and acetic anhydride, 15 μl. After 15 min at room temperature, volatiles were again removed with oxygen-free nitrogen. Tri-Sil Z (Pierce Chemical Co., Rockford, IL) (100 μl) was added to each vial and the mixtures were stirred for 1 hr at room temperature. Controls consisted of known glycoproteins and PVDF membrane.

Calcium binding

Calcium binding was assayed using a modification of a procedure from Maruyama *et al.* (1984). Protein fractions purified from the matrix were blotted on to nitrocellulose and dried at room temperature. The nitrocellulose was washed for 1 hr in 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole–HCl (pH 6.8). The membrane was washed for 15 min in the same buffer containing 5 mM $^{45}Ca^{2+}$ and rinsed with 50% EtOH. After drying, the membrane was placed in a film cassette with X-ray film and exposed for 24–48 hr before developing. Phosvitin (Sigma), an eggshell protein, was used as a positive control and aprotinin (Sigma), a protease inhibitor, as the negative control.

RESULTS

Total soluble protein content of the mussel shell is 0.3–0.5% by weight when determined by bicinchoninic acid assay. Insoluble material, determined to be 100% protein by elemental analysis (Table 1), accounts for 2–5% of the shell by weight; together, the soluble and insoluble proteins make up about

Table 1. Elemental analysis of mussel shell insoluble matrix

Sample No.	Carbon (%)	Hydrogen (%)	Nitrogen (%)
1	41.64	5.65	17.23
2	41.44	5.59	17.24
Total protein (average)	107%		

Total protein is calculated by using an average value of 6.25% nitrogen, which can vary with amino acid content, explaining why total protein is greater than unity

Table 2. Comparison of amino acid compositions total protein matrix from three molluscs

Amino acid	Total mussel matrix	Total nautilus matrix	Total abalone matrix
Gly	30.5	30.6	22.8
Ala	24.9	24.0	13.7
Ser	9.2	9.3	8.9
Asx	8.9	8.5	19.7
Leu	5.1	2.1	3.5
Glx	3.8	5.3	5.1
Arg	2.9	5.0	5.3
Lys	2.0	<1.0	2.2
Val	2.7	2.1	3.2
Phe	1.9	5.0	2.9
Tyr	2.2	1.5	3.0
Ile	1.6	1.5	1.4
Thr	1.3	1.5	2.4
Pro	1.2	1.3	4.1
Met	<1.0	<1.0	<1.0
His	<1.0	<1.0	<1.0
Cys	<1.0	<1.0	<1.0

Figures given are mol%, not adjusting for Trp, which cannot be detected by Pico-Tag analysis

3–5% of both the mussel and nautilus shells. The amino acid composition of the total protein extracted from the three shells is shown in Table 2. The data indicate that Gly, Ala, Ser, and Asx predominate, with Gly comprising the major mole percent in all cases. The Asx content is 8.5, 8.9, and 19.7% for nautilus, mussel, and abalone whole shell extract, respectively.

The soluble protein portion of the mussel matrix has six major protein components and a number of minor components. The major components were identified by SDS–PAGE; in the absence of reducing agents, the primary bands run at about 95, 45, 25, 21, 14 and 5 kDa (minor bands were present at 200, 185, 100, 69 and 19 kDa) (Fig. 1). Adding 2-mercaptoethanol to the sample buffer modifies many of the bands (Fig. 1). The four highest molecular weight bands were assayed and reacted with the sea urchin matrix antibody. Figure 2 illustrates a comparison of protein bands for the soluble proteins isolated from the mussel, nautilus, and abalone under non-reduced and reduced conditions. The principal bands from the nautilus were at 45, 40, 18, 15 and 3 kDa (minor band at 30 kDa) under non-reduced conditions, and from the abalone at 28 kDa (minor bands at 69, 38, 20, 17, 14, 7 and 5 kDa). In general, the bands were more diffuse from the abalone preparations than the nautilus and mussel.

Amino acid analysis of some of the soluble proteins from mussel shell is reported in Table 3. The lower molecular weight proteins have similar compositions with an abundance of Gly, Asx, and Ser residues, except for the 21 kDa protein, which also has a significant Phe and Lys component and the 45 kDa

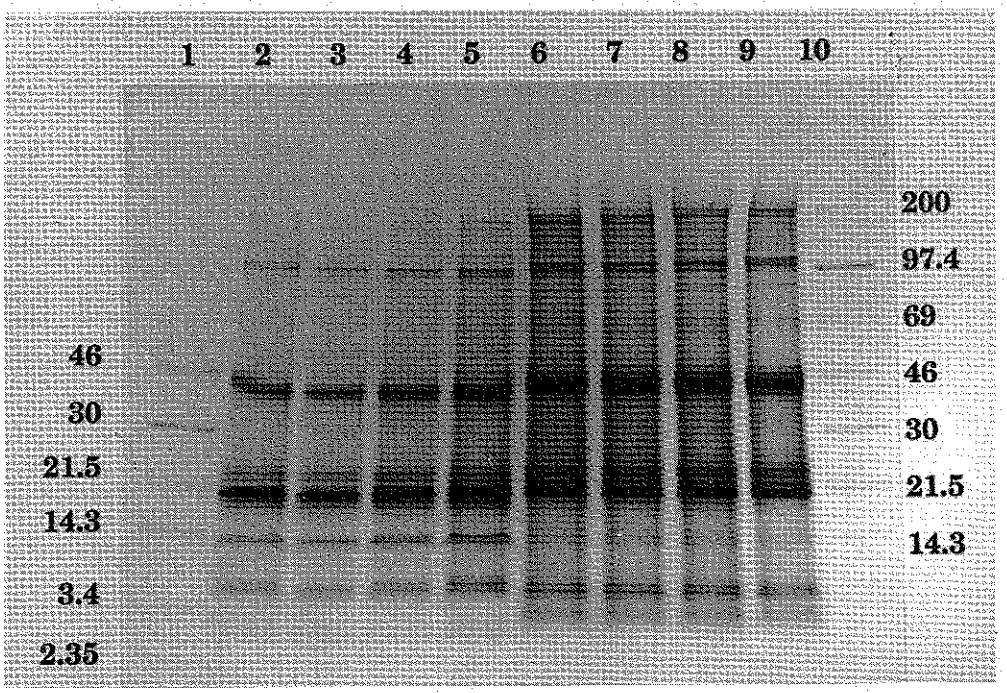

Fig. 1. Tricine SDS 10–20% gradient gel, 125 V, 90 min, 6 μg protein loaded per lane, stained with Coomassie Blue. Lanes 1 and 10 are Rainbow® molecular weight markers (Amersham, Arlington Heights, IL), sizes as indicated. Lanes 6–9 are run in the presence of 8% 2-mercaptoethanol, lanes 2–5 are run without 2-mercaptoethanol.

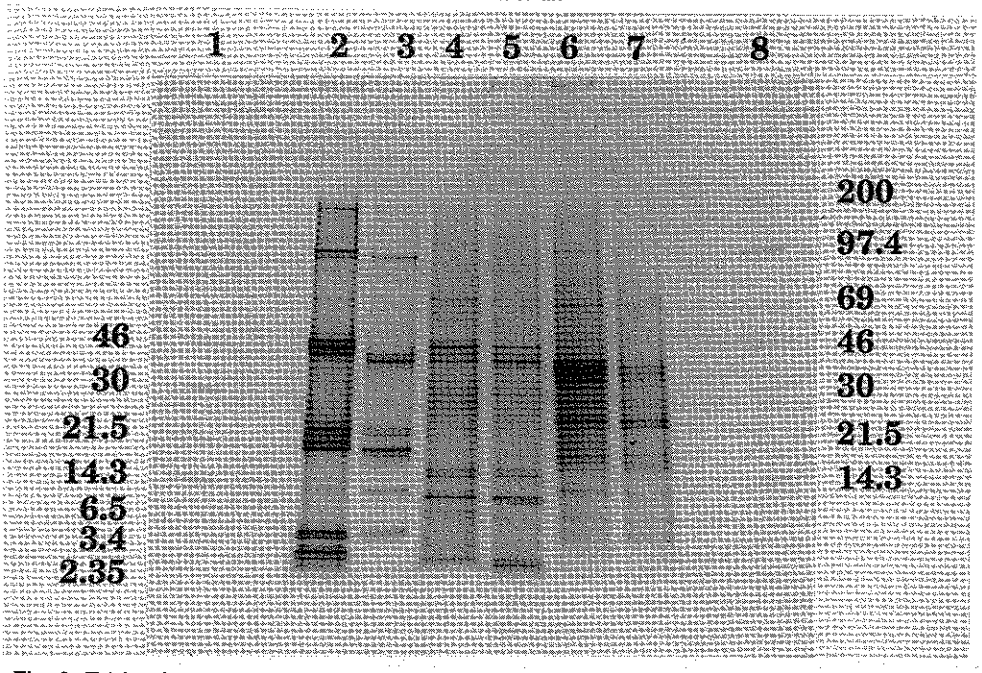

Fig. 2. Tricine SDS 10–20% gradient gel, 125 V, 90 min, 6 μg protein loaded per lane, stained with Coomassie Blue. Lanes 1 and 8 are Rainbow® molecular weight markers (Amersham, Arlington Heights, IL), sizes as indicated. Lane 2 is the mussel matrix reduced, lane 3 is the mussel matrix non-reduced, lane 4 is the nautilus matrix reduced, lane 5 is the nautilus matrix non-reduced, lane 6 is the abalone matrix reduced, and lane 7 is the abalone matrix non-reduced.

which has an abundance of Glx. The amino acid composition of the 95 and 200 kDa mussel proteins are very similar, with an abundance of Gly, Glx, and Asx. Analysis of glycosylation of the four highest molecular weight proteins isolated from the mussel shell soluble proteins found only the 95 kDa protein is glycosylated. This peptide contained *N*-acetyl glucosamine and glucose in an approximate ratio of 1:7 based on peak areas. Alcian Blue staining of the individual bands revealed no evidence for phosphorylated proteins.

Table 3. Amino acid composition of individual proteins from the mussel soluble matrix

	Molecular weight (kDa)						
Amino acid	200	95	45	21	14	5	SM50
Gly	14.0	8.5	13.0	19.7	26.3	26.3	22.2
Glx	9.4	9.1	10.1	4.8	4.3	5.8	13.8
Ser	8.2	8.2	7.6	9.5	11.2	10.1	3.8
Asx	11.9	15.4	10.7	8.8	7.7	8.1	8.8
Ala	7.1	6.4	4.9	5.3	5.9	6.8	4.7
Leu	5.9	8.0	6.1	5.1	6.8	8.1	1.8
Arg	5.8	5.6	5.5	3.6	5.7	4.5	6.5
Val	5.1	5.0	6.0	3.2	3.2	4.2	5.9
Lys	5.5	6.7	6.8	9.3	3.8	4.6	<1.0
Pro	7.0	5.1	6.8	7.2	4.7	3.6	14.5
Thr	6.5	8.1	6.6	5.2	4.0	3.7	1.8
Ile	4.3	5.7	4.6	4.5	5.6	5.2	1.4
Phe	2.7	3.3	3.4	10.1	4.5	3.9	3.8
His	1.1	<1.0	1.9	<1.0	1.0	1.0	0.0
Tyr*	2.7	1.9	4.6	2.5	3.2	2.7	2.0
Met*	2.5	1.8	<1.0	<1.0	<1.0	<1.0	5.4
Cys*	<1.0	<1.0	1.0	<1.0	1.3	1.3	1.1

Figures given are mole %, not adjusting for Trp, which cannot be detected by Pico-Tag analysis. *Indicates amino acids most sensitive to PVDF membrane procedure. Figures in last column are from published data on sea urchin SM50 protein given in mole percent and translated from genetic sequence data of Katoh-Fukui *et al.*, 1991.

The composition of the total soluble matrix of the three species (mussel, nautilus and abalone) (Table 4) shows a predominance of Gly, Asx, Ser, and Ala. There are some similarities to the sea urchin matrix in that Gly, Ser, Asx and Ala predominate, along with Glx. The soluble matrix from the abalone contains a high content of Asx, 41%, with a Asx plus Gly total of 76%. In comparison, the nautilus and mussel soluble matrices contain only 13 and 14.6% Asx, respectively.

N-terminal sequences of the 45, 21 and 5 kDa bands are reported later (two letters separated by a

Table 4. Comparison of amino acid compositions of soluble matrices from the three molluscs

Amino acid	Soluble mussel matrix	Soluble nautilus matrix	Soluble abalone matrix	Soluble sea urchin spicule
Gly	30.0	31.3	35.5	21.3
Ala	16.8	13.2	2.9	8.3
Ser	9.9	9.6	3.4	12.5
Asx	14.6	13.0	41.0	11.0
Leu	3.8	2.9	<1.0	4.2
Glx	5.0	6.6	3.1	12.1
Arg	2.3	2.8	1.9	2.3
Lys	2.5	1.5	2.9	3.3
Val	2.1	2.6	1.1	3.4
Phe	1.5	3.4	<1.0	1.8
Tyr	2.7	2.2	<1.0	1.3
Ile	1.1	1.2	<1.0	2.5
Thr	1.6	3.4	2.3	4.4
Pro	2.6	4.3	2.3	4.5
Met	2.7	<1.0	<1.0	<1.0
His	<1.0	<1.0	<1.0	3.7
Cys	<1.0	<1.0	<1.0	2.9

Figures given are mole %, not adjusting for Trp, which cannot be detected by Pico-Tag analysis. Comparative data for the sea urchin, *Strongtyocentrotus purpuratus* is from Benson *et al.*, 1986.

slash indicates ambiguity in the sequence). The 14 kDa band consisted of more than one peptide, but the dominant sequence was identical to residues 1–9 of the 21 kDa band. None of these sequences has significant homology with the sea urchin SM50 protein (Benson *et al.*, 1986; Katoh-Fukui *et al.*, 1991). most prominent periostracal bands are at 35 and 16 kDa under non-reducing conditions, and 35, 16 and 10 kDa when reduced. Also, the amino acid composition of the isolated proteins bear no resemblance to that reported for periostracum (Waite *et al.*, 1979).

45 kDa: V–G–Y–M–G–V–R–P–P–S–L–K–Q

21 kDa: K–H–F–A–F–F–G–Q–P–S–Y–N–A–F–N–R–N–K–F–M–T–D–F–M–T–T–F–N–Q–I

5 kDa: A/F–N–T/G–D–R–N–D–L–L–A–V–A–G/K–L.

Extraction of the insoluble portion of the matrix by grinding, boiling and subsequent centrifugation yields additional soluble material with the same amino acid composition as that described above for the soluble matrix in Table 4. Some of the matrix remains insoluble even after these additional treatments. The amino acid compositions of the insoluble matrix from mussel, nautilus and abalone (Table 5) indicate a predominance of Gly, Ala, and Ser residues similar to the 62.6% reported for insoluble spider dragline silk (Table 4). The abalone insoluble matrix contains 19.2% Asx. No chitin was detected in the insoluble material from the mussel or abalone, or the whole shell from the mussel or abalone, by hydrolysis and gas chromatographic analysis for *N*-acetyl glucosamine. Chitin, as *N*-acetyl glucosamine, was detected in the nautilus shell and a shrimp shell control (Fig. 3).

None of the mussel shell proteins isolated is a periostracal proteins. This was precluded by removal of the outer shell layer during preparation of the shell material for purification of matrix. A control preparation of periostracum scraped from the outer shell revealed bands which do not correspond to any of the proteins purified from the matrix. The

Table 5. Comparison of amino acid compositions of insoluble matrices from the three molluscs

Amino acid	Insoluble mussel matrix	Insoluble nautilus matrix	Insoluble abalone matrix	Insoluble spider silk
Gly	30.7	31.9	22.0	37.1
Ala	25.2	22.9	13.3	21.1
Ser	9.5	9.4	7.3	4.5
Asx	7.5	8.6	19.2	2.5
Leu	5.8	2.1	3.1	3.8
Glx	3.5	5.3	5.3	9.2
Arg	2.9	5.1	5.5	7.6
Lys	1.9	<1.0	2.2	<1.0
Val	2.8	2.1	3.5	1.8
Phe	2.0	4.8	3.2	—
Tyr	2.1	1.6	4.0	2.9
Ile	1.9	1.3	1.7	<1.0
Thr	1.4	1.6	2.4	1.7
Pro	1.3	1.4	5.2	4.3
Met	<1.0	<1.0	<1.0	<1.0
His	<1.0	<1.0	<1.0	—
Cys	<1.0	<1.0	<1.0	<1.0

Figures given are mole %, not adjusting for Trp, which cannot be detected by Pico-Tag analysis. Comparative data for the spider dragline silk, *Nephila clavipes*, is from Lombardi and Kaplan (1990).

Both total soluble matrix and total insoluble matrix from the mussel bind calcium under the assay conditions studied. The method of preparation was with acetic acid since artifacts due to EDTA extraction methods have been reported (Weiner, 1979; Wheeler *et al.*, 1987). None of the individual proteins isolated from the matrix bound calcium under the assay conditions, the 95, 45, 21, 14 and 5 kDa bands gave a positive (blue) response to differential staining with Stains-All, sensitive to calcium binding. The proteins used as molecular weight markers gave a negative (pink) response. The 200 kDa band did not stain at all with Stains-All, but did stain with Coomassie Blue.

DISCUSSION

No glucosamine or *N*-acetyl glucosamine residues, which would indicate the presence of chitin or chitosan, were detected during GLC analysis of the mussel shell insoluble material, or the powdered shell itself. This supports the conclusion from the elemental analysis that the matrix is composed almost completely of protein.

The molecular weight patterns and the modification of the bands upon addition of 2-mercaptoethanol to the sample buffer suggest that some of the proteins isolated from the mussel shell matrix may consist of subunits of the smaller proteins. Amino acid analysis of the 200 and 95 kDa proteins also suggests they are similar and further supports the subunit hypothesis. The identical partial sequence data collected for the 21 and 14 kDa proteins also indicate possible subunits of smaller protein peptides.

Amino acid analyses of some fractions of the molluscan matrix have been reported. These include analyses by Hare and Abelson (1965) (*Mercenaria mercenaria*, *Mytilus californianus*, *Mytilus edulis*, *Mytilus veridus*, *Protothaca grata*, *Sanguinolaria nuttalli*, *Tagelus californianus* and *Tivella argentina*); Meenakshi *et al.* (1971, 1975) (*Campeloma decisum*, *Littorina irrorata*, *Nassarius obsoletus*, *Pila virens*, *Polinices duplucatus*, *Pomacea paludosa* and *Thais floridana*); Crenshaw (1972) (*Mercenaria mercenaria*); Weiner and Hood (1975) (*Crassostrea irredescens*, *Crassostrea virginica*, *Mercenaria mercenaria* and *Nautilus pompilius*); Weiner *et al.* (1976)

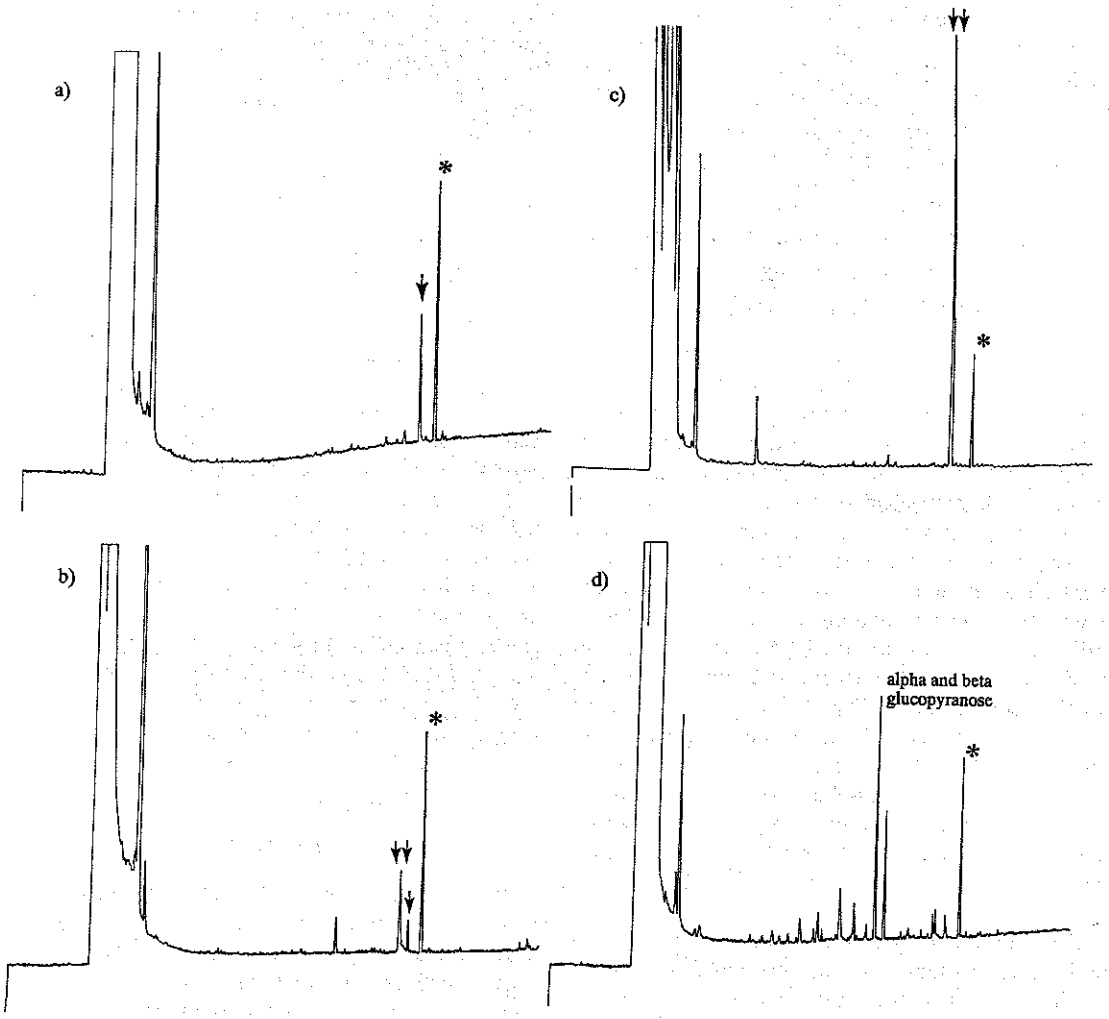

Fig. 3. Gas-chromatographic analysis of (a) *N*-acetyl glucosamine standard, (b) insoluble nautilus matrix, (c) pre-hydrolyzed shrimp chitin, and (d) insoluble mussel matrix. Mesoinositol (*) is used as the internal standard (14.28 min). A single arrow indicates the position of the *N*-acetyl glucosamine peak (13.89).

(*Neotrigonia margaritacea* and *Scabrotrigonia thoracica*); Weiner (1982) (*Nautilus repertus*); Krampitz *et al.* (1983a) (*Crassostrea gigas*); and Wheeler and Sikes (1989) (*Crassostrea virginica*). Unlike the data reported here, none of these analyses are on individual proteins isolated from the organic matrix present in the shell.

The composition of the total soluble matrix (Table 3) bears some similarity to the spicule matrix from *Strongylocentrus purpuratus* (Benson *et al.*, 1986), especially in the percentages of Gly, Ala, Ser, Asx, and Glx, which total 65.2%. The amino acid composition of some of the 95 through 5 kDa proteins suggests that they may be the acidic fraction often referred to in the literature as having an influence on crystallization (Weiner, 1981; Addadi and Weiner, 1985, 1986; Berman *et al.*, 1988). Some researchers report difficulty in staining these types of acidic proteins (Weiner, 1982); however, when poor staining was observed in the current study it was often attributable to insufficient protein concentration. Butler *et al.* (1981) was able to stain acidic rat glycoproteins with Coomassie Blue and Weiner *et al.* (1977) had earlier reported staining of *Mytilus* proteins with Coomassie Blue. Comparison of the isolated proteins with the 50 kDa protein from sea urchin spicules sequenced by Sucov *et al.* (1987) and corrected in Katoh-Fukui *et al.* (1991) (Table 2) shows few similarities. Of particular note is the absence of a high percentage of proline reported in the sea urchin protein.

Comparison of the two unambiguous sequences (45 and 21 kDa proteins) on Lasergene® software (DNASTAR Inc., Madison, WI), especially with Kyte–Doolittle hydrophobicity plots, suggests that these may be membrane proteins, although the sequence examined is too short for any conclusive determination (Fig. 3). At least with the 21 kDa peptide a hydrophobic domain of 14 amino acids is noted, although this is still not of sufficient length to

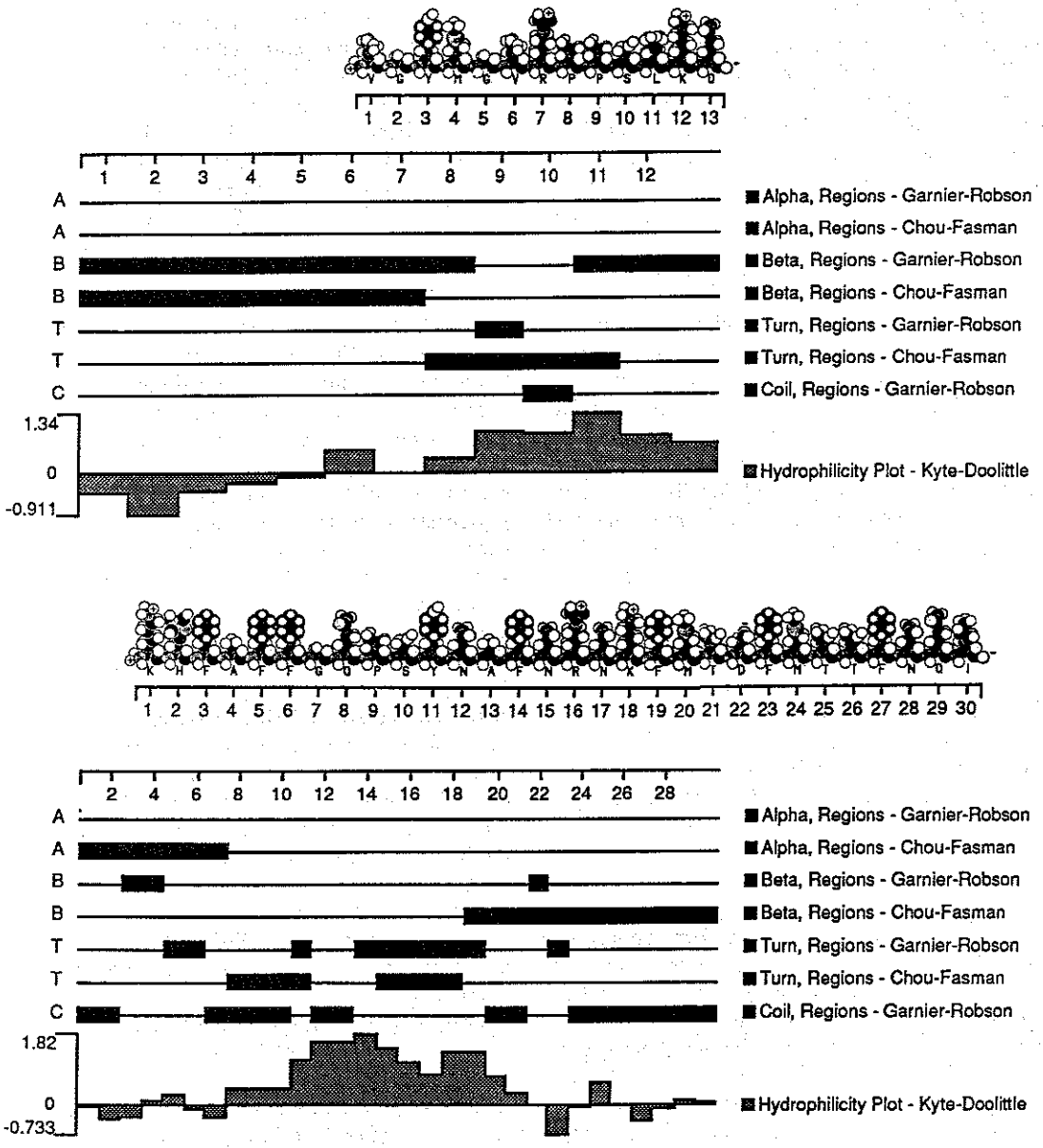

Fig. 4. Lasergene® analysis of protein sequences. Top sequence is the 45 kDa protein, bottom is the 21 kDa protein.

form a transmembrane segment. Membrane proteins would be consistent with a mineralization model in which crystallization occurs within vesicles at the mineralization front or surface. Also, some extended beta-sheet secondary structure domains are evident in these analyses.

The amino acid composition of the insoluble matrices indicates a predominance of Gly, Ala, and Ser, which is characteristic of silks. A comparison of the amino acid composition of the insoluble matrix to spider dragline silk indicates strong similarities (Table 5). These data are also in good agreement, taking into account differences in methodology, with the amino acid composition reported by Hare and Abelson (1965) for the insoluble *M. edulis* matrix; no previous reference to amino acid analysis of insoluble *N. pompilius* matrix was found. A beta-sheet structure has been suggested as a key scaffolding element in the organic matrix of the mollusc shell based on X-ray diffraction analysis (Weiner and Traub, 1980, 1981), and the composition of the mussel, nautilus, and abalone shell insoluble matrices reported here supports this hypothesis. The finding that the soluble and insoluble matrices have similar amino acid compositions, coupled with the fact that, with additional treatments, additional soluble material was released from the insoluble fraction, may indicate that these are, in fact, the same material.

If we assume that the bulk of the Asx (8–11%) is Asp, then this suggests a role for anionic proteins in binding calcium in both the total soluble and insoluble matrix. It should be noted that the assay used characterizes calcium affinity of proteins immobilized on membranes, which may be different from calcium affinity of protein free in solution (Wheeler and Sikes, 1989). In addition, the assay does not consider interactions with other macromolecules which may be present *in vivo*. This immobilization assay may still be more representative of *in vivo* conditions than free solution measurements if immobilized proteins are responsible for the initiation of crystallization, as suggested by Linde *et al.* (1989). Aside from differences in free solution vs immobilized conditions for the assay, subtle changes in protein conformation under the assay conditions could also impact on calcium binding affinity. Additionally, our initial studies on calcium binding were carried out with protein fractions that had been isolated from mollusc shell matrices using the traditional EDTA methods. However, subsequent analysis of the purified protein fractions by mass spectroscopy revealed residual EDTA bound to the proteins which could confound calcium binding studies. This potential problem was eliminated by using the acetic acid preparations.

Chitin is postulated to be a key structural element and the primary polysaccharide in molluscan shells, according to biomineralization theories (Lowenstam and Weiner, 1989; Wheeler and Sikes, 1989; Simkiss and Wilbur, 1989). Chitin is proposed in these theories to function as an insoluble scaffolding element, which interacts with proteins to form appropriate molecular recognition sites for initiating crystallization of calcium carbonate. We find no evidence for a significant chitin structural element in two of these composite shells (mussel and abalone) after characterization by chromatographic techniques. The only evidence for chitin in these systems is *N*-acetyl glucosamine derivatives in the nautilus shell and trace levels of glucose and *N*-acetyl glucosamine derivatives associated with some glycoproteins in the soluble portion of the mussel shell macromolecular organic matrix.

Initial studies reporting chitin as a component in mollusc shells used indirect measures for identifying *N*-acetyl glucosamine, including enzymatic and staining methods (Jeuniaux, 1963; Peters, 1972; Goffinet and Jeuniaux, 1979). No detailed chromatographic study has been conducted to describe more fully the polysaccharide components and their function in these systems. In this study, no *N*-acetyl glucosamine derivative was detected in the insoluble mussel (*M. edulis*) matrix, insoluble abalone (*H. rufescens*) matrix, powdered whole mussel shell, or the residue left after treating *M. edulis* matrix for the semimicro estimation of chitin (Hackman and Goldberg, 1971), which is presumed by the assay to be chitin. An *N*-acetyl glucosamine derivative peak did appear in both the prehydrolyzed shrimp chitin (positive control) and the *N. pompilus* shell as reported by Weiner and Traub (1980). The lack of detection of an *N*-acetyl glucosamine derivative in these analyses is strong evidence for the absence of chitin.

The absence of chitin in our analysis of mollusc shells is partly supported by Peters (1972), who found no chitin in *M. edulis* shell, but did find chitin in *Haliotis lamellosa*, the only abalone species he reported. He did not examine a *Nautilus* species. Peters used KOH hydrolysis, followed by an iodine color test of the reaction products. Richards (1951) indicated that this test is sensitive to the degree of hydrolysis, making the results variable on the same substrate. Goffinet and Jeuniaux (1979) report chitin in *M. edulis*, *N. pompilius* and *H. tuberculata*, using an enzymatic method (Jeuniaux, 1966). A close examination of this method reveals that it relies on β-glucosidase, which in pure preparation should not degrade chitin (personal communication, Elwyn Reese, this center), or lobster blood serum, where the concentration of chitin-degrading enzymes is unknown, to digest chitin for use in a colorimetric assay for hexosamines. This particular colorimetric assay gives false positives in the presence of certain amino acids, particularly L-lysine (Dische, 1962). Amino acid analysis of *M. edulis* samples isolated in the present study shows lysine composed 1.9 (insoluble) and 2.5 (soluble) mole percent of the matrix. Therefore, it is not surprising that the assay gives a positive result with every calcified layer of mollusc shell examined, or about 150 species (Poulicek *et al.*, 1986), since virtually 100% of the macromolecular matrix is protein containing lysine. Dische (1962) recommends gel filtration chromatography to eliminate this interference.

These findings call into question the premise that a polysaccharide scaffolding is involved, along with proteins, in biomineralization processes in all molluscs. It is possible that chitin scaffolding may exist in some species, specifically the *Nautilus* (Class Cephalopda); however, it should not be considered a necessary or universal component of mineralizing matrices, as both the *Mytilus* (Class Pelecypoda) and *Haliotis* (Class Gastropoda) shells contain nacre, but no chitin. *In vivo* conditions for mineralization may be more complex than suggested by models of matrix-mediated crystallization, which assign structural support to a chitin-based polysaccharide coupled to a silk-like protein component (Weiner *et al.*, 1983; Weiner and Traub, 1984). In addition, the finding of similarities in composition between the soluble and insoluble protein fractions raise questions concerning the distinctions often ascribed to these fractions in most mineralization models; insoluble scaffolding proteins and soluble binding/recognition proteins. Further research on the structural, temporal and molecular recognition issues associated with the matrix macromolecules may reveal that more complex interactions, such as between several organic

components, may be required to mediate controlled crystal growth in molluscan shells. This level of understanding may be required before biomimetic approaches to this process can be fully realized.

Acknowledgements—We thank Hap Wheeler, Richard Laursen, Steve Benson, and Stephen Weiner for helpful discussions during the course of these studies. Our thanks to Memet Sarikaya for supplying the nautilus shell and Steve Benson for the antibody.

REFERENCES

Addadi L. and Weiner S.(1985) Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization. *Proc. natn. Acad. Sci. U.S.A.* **82,** 4110–4114.

Addadi L. and Weiner S. (1986) Interactions between acidic macromolecules and structured crystal surfaces: stereochemistry and biomineralization. *Mol. Cryst. Liq. Cryst.* **13,** 305–322.

Beedham G. E. (1958) Observations on the non-calcareous component of the shell of the Lamellibranchia. *Q. J. microsc. Sci.* **99,** 341–357.

Benson S. C., Benson N. C. and Wilt F. (1986) The organic matrix of the skeletal spicule of sea urchin embryos. *J. Cell Biol.* **102,** 1878–1886.

Berman A., Addadi L. and Weiner S. (1988) Interactions of sea urchin skeleton macromolecules with growing calcite crystals—a study of intracrystalline proteins. *Nature* **331,** 546–548.

Butler W. T., Bhown M., Dimuzio M. T. and Linde A. (1981) Noncollagenous proteins of dentin. Isolation and partial characterization of rat dentin proteins and proteoglycans using a three-step preparative method. *Coll. Res.* **1,** 187–199.

Carriker M. R. (1978) Ultrastructural analysis of dissolution of shell of the bivalve *Mytilus edulis* by the accessory boring organ of the gastropod *Urosalpinx cinerea*. *Mar. Biol.* **48,** 105–134.

Campbell K. P., MacLennan D. H. and Jorgensen A. O. (1983) Staining of the Ca^{2+}-binding proteins, calsequestrin, calmodulin, troponin C and S-100, with the cationic carbocyanine dye "Stains-all". *J. biol. Chem.* **258,**11267–11273.

Chaplin M. F. (1982) A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas–liquid chromatography. *Analyt. Biochem.* **123,** 336–341.

Cohen S. A., Meys M. and Tarvin T. L. (1989) *The Pico-Tag® Method, A Manual of Advanced Techniques for Amino Acid Analysis.* Waters Division of Millipore, Milford, MA.

Crenshaw M. A. (1972) The soluble matrix from *Mercenaria mercenaria* shell. *Biomineralization* **6,** 6–11.

Crenshaw M. A. and Ristedt H. (1976) The histochemical localization of reactive groups in septal nacre from *Nautilus pompilus* L. In *The Mechanisms of Mineralization in the Invertebrates and Plants* (Edited by Watabe N. and Wilbur K. M.), pp. 355–367. University of South Carolina Press, SC.

Dische Z. (1962) Color reactions of hexosamines. In *Methods in Carbohydrate Chemistry* (Edited by Whistler R. L. and Wolfram M. L.), Vol. 1, pp. 507–512. Academic Press, New York.

Furlong C. E. and Humbert R. (1992) Design of protein-producing bioreactors for self-assembling systems. *Mat. Res. Soc. Symp. Proc.* **255,** 435–442.

Goffinet G. and Jeuniaux C. (1979) Distribution et importance quantitative de la chitine dans les coquilles de mollusques. *Cah. Biol. Mar.* **20,** 341–349.

Gordon J. and Carriker M. R. (1980) Sclerotized protein in the shell matrix of a bivalve mollusc. *Mar. Biol.* **57,** 251–260.

Gregoire C. (1961) Structure of the conchiolin cases of the prisms in *Mytilus edulis* Linne. *J. biophys. biochem. Cytol.* **9,** 395–400.

Gregoire C. L. (1972) Structure of the molluscan shell. In *Chemical Zoology* (Edited by Florkin M. and Scheer B. T.), Vol. 7, pp. 45–102. Academic Press, New York.

Hackman R. H. and Goldberg M. (1971) Studies on the hardening and darkening of insect cuticles. *J. Insect Physiol.* **17,** 335–347.

Hare P. E. and Abelson P. H. (1965) Amino acid composition of some calcified proteins. *Carnegie Institution Yearbook* **64,** 223–232.

Jeuniaux C. (1963) *Chitine et Chitinolyse.* Masson, Paris, France.

Jeuniaux C. (1966) Chitinases. In *Methods in Enzymology* (Edited by Neufeld E. F. and Ginsburg V.), Vol. 8. Academic Press, New York.

Katoh-Fukui Y., Noce T., Ueda T., Fujiwara Y., Hashimoto N., Higashinakagawa T., Killian C., Livingston B. T., Wilt F. H., Benson S. C., Sucov H. M. and Davidson E. H. (1991) The corrected structure of the SM50 spicule matrix protein of *Strongylocentrotus purpuratus. Devl. Biol.* **145,** 201–202.

Kennedy W. J., Taylor J. D. and Hall A. (1969) Environmental and biological controls on bivalve shell mineralogy. *Biol. Rev.* **44,** 499–530.

Krampitz G., Drolshagen H., Hausle J. and Hof-Irmscher K. (1983a) Organic matrices of mollusc shells. In *Biomineralization and Biological Metal Accumulation* (Edited by Westbroek P. and DeJong E. W.), pp. 231–247. D. Reidel, Dordrecht, Holland.

Krampitz G., Drolshagen H. and Hotta S. (1983b) Simultaneous binding of calcium and bicarbonate by conchiolin of oyster shells. *Experientia* **39,** 1104–1105.

Krampitz G., Engels J. and Cazaux C. (1976) Biochemical studies on water-soluble proteins and related components of gastropod shells. In *The Mechanisms of Mineralization in Invertebrates and Plants* (Edited by Watabe N. and Wilbur K. M.), pp. 155–173. University of South Carolina Press, South Carolina.

LeGendre N. and Matsudaira P. (1988) Direct protein microsequencing from Immobilon-P Transfer Membrane. *Biotechnology* **6,** (2) 154–159.

Linde A., Lussi A. and Crenshaw M. A. (1989) Mineral induction by immobilized polyanionic proteins. *Calcif. Tissue Int.* **44,** 286–295.

Lombardi S. and Kaplan D. (1990) The amino acid composition of major ampullate gland silk (dragline) of *Nephila clavipes* (Araneae, Tetragnathidae). *J. Arachnol.* **18,** 297–306.

Lowenstam H. A. and Weiner S. (1989) *On Biomineralization.* Oxford University Press, New York.

Maruyama K., Mikawa T. and Ebashi S. (1984) Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem.* **95,** 511–519.

McKeon T. A. and Lyman M. L. (1991) Calcium ion improves electrophoretic transfer of calmodulin and other small proteins. *Analyt. Biochem.* **193,** 125–130.

Meenakshi V. R., Blackwelder P. L., Hare P. E., Wilbur K. M. and Watabe N. (1975) Studies on shell regeneration I. Matrix and mineral composition of the normal and regenerated shell of *Pomacea paludosa. Comp. Biochem. Physiol.* **50A,** 347–351.

Meenakshi V. R., Hare P. E. and Wilbur K. M. (1971) Amino acids of the organic matrix of neogastropod shells. *Comp. Biochem. Physiol.* **40B,** 1037–1043.

Peters W. (1972) Occurrence of chitin in Mollusca. *Comp. Biochem. Physiol.* **41B,** 541–550.

Poulicek M., Voss-Foucart M. F. and Jeuniaux C. (1986) Chitinoproteic complexes and mineralization in mollusk skeletal structures. In *Chitin in Nature and Technology* (Edited by Muzzarelli R., Jeuniaux C. and Gooday G.). Plenum Press, New York.

Richards A. G. (1951) *The Integument of Arthropods*. University of Minnesota Press, Minneapolis, MN.

Sikes C. S. and Wheeler A. P. (1983) A systematic approach to some fundamental questions of calcium carbonate calcification. In *Biomineralization and Biological Metal Accumulation* (Edited by Westbroek P. and DeJong E. W.), pp. 285–289. D. Reidel, Dordrecht, Holland.

Simkiss K. (1965) The organic matrix of the oyster shell. *Comp. Biochem. Physiol.* **16,** 427–435.

Simkiss K. and Wilbur K. (1989) *Biomineralization: Cell Biology and Mineral Deposition*. Academic Press, San Diego, CA.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J. and Klenk D. C. (1985) Measurement of protein using bicinchoninic acid. *Analyt. Biochem.* **150,** 76–85.

Sucov H. M., Benson S., Robinson J. J., Britten R. J., Wilt F. and Davidson E. H. (1987) A lineage-specific gene encoding a major matrix protein of the sea urchin embryo spicule. *Devl. Biol.* **120,** 507–519.

Swift D. M., Sikes C. S. and Wheeler A. P. (1986) Analysis and function of organic matrix from sea urchin tests. *J. exp. Zool.* **240,** 65–73.

Veis D. J., Albinder T. M., Clohisy J., Rahima M., Sabsay B. and Veis A. (1986) Matrix proteins of the teeth of the sea urchin *Lytechinus variegatus*. *J. exp. Zool.* **240,** 35–46.

Waite J. H., Saleuddin A. S. M. and Andersen S. O. (1979) Periostracin—a soluble precursor of sclerotized periostracum in *Mytilus edulis* L. *J. comp. Physiol.* **130,** 301–307.

Weiner S. (1979) Aspartic acid-rich proteins: major components of the soluble organic matrix of mollusk shells. *Calcif. Tissue Int.* **29,** 163–167.

Weiner S. (1981) Repeating amino acid sequences in the soluble protein of mollusk shell organic matrices: their involvement in crystal formation. In *The Chemistry and Biology of Mineralized Connective Tissues* (Edited by Veis A.), pp. 517–521. Elsevier, Amsterdam.

Weiner S. (1982) Separation of acidic proteins from mineralized tissues by reversed-phase high-performance liquid chromatography. *J. Chromat.* **245,** 148–154.

Weiner S. and Hood L. (1975) Soluble protein of the organic matrix of mollusc shells: a potential template for shell formation. *Science* **190,** 987–989.

Weiner S., Lowenstam H. A. and Hood L. (1976) Characterization of 80-million-year-old mollusk shell proteins. *Proc. natn. Acad. Sci. U.S.A.* **73,** (8) 2541–2545.

Weiner S., Lowenstam H. A. and Hood L. (1977) Discrete molecular weight components of the organic matrices of mollusc shells. *J. exp. Mar. Biol. Ecol.* **30,** 45–51.

Weiner S. and Traub W. (1980) X-Ray diffraction study of the insoluble organic matrix of mollusc shells. *FEBS Lett.* **111,** (2) 311–316.

Weiner S. and Traub W. (1981) Organic–matrix–mineral relationships in mollusk-shell nacreous layers. In *Structural Aspects of Recognition and Assembly in Biological Macromolecules* (Edited by Balaban M., Sussman J. L., Traub W. and Yonath A.), pp. 467–482. Balaban ISS.

Weiner S. and Traub W. (1984) Macromolecules in mollusc shells and their functions in biomineralization. *Phil. Trans. R. Soc. Lond. Ser. B.* **304,** 421–438.

Weiner S., Traub W. and Lowenstam H. A. (1983) Organic matrix in calcified exoskeletons. In *Biomineralization and Biological Metal Accumulation* (Edited by Westbroek P. and DeJong E. W.), pp. 205–224. D. Reidel, Dordrecht, Holland.

Wheeler A. P., Rusenko K. W., George J. W. and Sikes C. S. (1987) Evaluation of calcium binding by molluscan shell organic matrix and its relevance to biomineralization. *Comp. Biochem. Physiol.* **87B,** 953–960.

Wheeler A. P. and Sikes C. S. (1989) Matrix–crystal interaction in $CaCO_3$ biomineralization. In *Biomineralization Chemical and Biochemical Perspectives* (Edited by Mann S., Webb J. and Williams R. P.), pp. 95–132. VCH, New York.

Wilbur K. (1964) Shell formation and regeneration. In *Physiology of Mollusca* (Edited by Wilbur K. M. and Yonge C. M.), Vol. 1, pp. 243–282. Academic Press, New York.